Propofol Increases Phosphorylation of Troponin I and Myosin Light Chain 2 via Protein Kinase C Activation in Cardiomyocytes

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Background: Troponin I (TnI) and myosin light chain 2 (MLC2) are important myofibrillar proteins involved in the regulation of myofilament calcium (Ca²⁺) sensitivity and cardiac inotropy. The objectives of this study were to determine the role of protein kinase C (PKC) in mediating propofol-induced changes in actomyosin adenosine triphosphatase activity in cardiac myofibrils and to examine the extent to which propofol alters the phosphorylation of TnI and MLC2 in cardiomyocytes.

Methods: Freshly isolated adult rat ventricular myocytes were used for the study. Cardiac myofibrils were extracted for assessment of actomyosin adenosine triphosphatase activity and phosphorylation of TnI and MLC2. Western blot analysis for PKC-α was performed on cardiomyocyte subcellular fractions. Simultaneous measurement of intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) and myocyte shortening was assessed using fura-2 and video edge detection, respectively.

Results: Propofol (30 μM) reduced the Ca²⁺ concentration required for activation of actomyosin adenosine triphosphatase activity, and this effect was abolished by bisindolylmaleimide I. In addition, propofol stimulated dose-dependent phosphorylation of TnI and MLC2. PKC activation with phorbol myristic acetate also stimulated an increase in phosphorylation of TnI and MLC2. The actions of propofol and phorbol myristic acetate together on phosphorylation of TnI and MLC2 were not additive. PKC inhibition with bisindolylmaleimide I attenuated phorbol myristic acetate– and propofol-induced phosphorylation of TnI and MLC2. Propofol stimulated translocation of PKC-α from cytosolic to membrane fraction. Propofol caused a shift in the extracellular Ca²⁺–shortening relationship, and this effect was abolished by bisindolylmaleimide I.

Conclusions: These results suggest that propofol increases myofilament Ca²⁺ sensitivity via a PKC-dependent pathway involving the phosphorylation of MLC2.

ALTERATIONS in cardiac inotropy and lusitropy are mediated by changes in the availability of intracellular free calcium (Ca²⁺) concentration ([Ca²⁺]ᵢ) and/or myofilament Ca²⁺ sensitivity. The sensitivity of the cardiac myofilaments to [Ca²⁺]ᵢ is primarily regulated by the phosphorylation state of troponin I (TnI) and myosin light chain 2 (MLC2). However, changes in intracellular pH

are also known to modulate myofibrillar responsiveness to Ca²⁺.[1] Previous studies have demonstrated that activation of protein kinase C (PKC) in rat cardiomyocytes results in phosphorylation of several myofibrillar proteins and an increase in myofilament Ca²⁺ sensitivity.[2–5] An increase in intracellular pH is also observed in response to PKC activation.[6,7] Therefore, PKC activation can trigger several cellular mechanisms that can increase myofilament Ca²⁺ sensitivity, resulting in changes in cardiac inotropy and lusitropy.

We previously demonstrated that propofol causes a negative inotropic effect in rat ventricular myocytes under baseline conditions[8] as well as during activation of sympathetic β adrenoreceptors.[9] However, several recent studies from our laboratory and others have also shown that propofol can increase myofilament Ca²⁺ sensitivity in cardiac muscle.[7,8,10,11] This propofol-induced increase in myofilament Ca²⁺ sensitivity acts to offset the negative inotropic action of propofol.[8] The propofol-induced increase in myofilament Ca²⁺ sensitivity in rat cardiomyocytes was associated, in part, with intracellular alkalinization mediated via PKC-dependent activation of sodium ion–hydrogen ion (Na⁺–H⁺) exchange.[7] The propofol-induced increase in myofilament Ca²⁺ sensitivity is only partially attenuated by inhibition of Na⁺–H⁺ exchange,[7] suggesting that additional mechanisms could be involved in the propofol-induced increase in myofilament Ca²⁺ sensitivity. One possibility is that propofol alters PKC-dependent phosphorylation of the cardiomyocyte contractile proteins, TnI and/or MLC2.

The goals of the current study were to assess the effects of PKC inhibition on propofol-induced alterations in myofilament Ca²⁺ sensitivity and to correlate this effect with the phosphorylation state of cardiomyocyte myofibrillar proteins. We tested the hypothesis that propofol alters the phosphorylation state of TnI and MLC2 via a PKC-dependent pathway.

Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee (Cleveland, Ohio).

Ventricular Myocyte Preparation

Adult ventricular myocytes from rat hearts were isolated as previously described using established proce-

dures.[9] In brief, the hearts were excised, cannulated *via* the aorta, attached to a modified Langendorff perfusion apparatus, and perfused with oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit buffer (37°C) containing the following: 118 mM sodium chloride, 4.8 mM potassium chloride, 1.2 mM magnesium chloride ($MgCl_2$), 1.2 mM potassium phosphate (KH_2PO_4), 1.2 mM calcium chloride, 37.5 mM sodium bicarbonate, 16.5 mM dextrose; pH 7.35. Following a 5-min equilibration period, the perfusion buffer was changed to a Ca^{2+}-free Henseleit buffer containing 40 mg collagenase type II (Worthington Biochemical Corp., Freehold, NJ; Lot No. 40P4421, 268 U/ml). Following collagenase digestion (20 min), the ventricles were minced and shaken in Henseleit buffer. The resulting cellular digest was washed, filtered using cheesecloth to trap any undigested cardiac tissue, and resuspended in phosphate-free HEPES-buffered saline (HBS) containing the following: 118 mM sodium chloride, 4.8 mM potassium chloride, 1.2 mM $MgCl_2$, 1.25 mM calcium chloride, 11 mM dextrose, 25 mM HEPES, 5 mM pyruvate; pH 7.35, and vigorously bubbled immediately before use with 100% O_2. Typically, $6-8 \times 10^6$ cells per rat heart were obtained using this procedure. Viability, as assessed by the percentage of cells retaining a rod-shaped morphology, was routinely between 80 and 90%.

Preparation of Cardiac Myofibrils

Two-milliliter aliquots of the freshly isolated myocyte suspension were incubated in the presence or absence of propofol for 10 min at 37°C with gentle agitation. The myocytes were immediately washed twice in ice-cold HBS containing protease and phosphatase inhibitors and pelleted at 400g for 3 min, after which an equivalent volume of extraction buffer, 50 mM Tris(hydroxymethyl)-aminomethane (pH 7.5) containing Triton X-100 (0.1%), sodium fluorescein (20 mM), dithiothreitol (0.5 mM), $MgCl_2$ (0.5 mM), EDTA (0.125 mM), antipain (5 μg/ml), leupeptin (10 μg/ml), pepstatin A (5 μg/ml), and para-methylsulfonic acid (43 μg/ml) was added to the suspension. The cells were homogenized and kept on ice for 30 min. The Triton-extracted myofibrils were pelleted at 10,000g (5 min, 4°C). The detergent solubilized supernatant was set aside; the pellet was resuspended in an equivalent volume of extraction buffer and washed twice again. The resultant myofibrillar fraction was resuspended in Ca^{2+}-free extraction buffer and stored at −20°C. Examination of the pellet under the microscope indicated that it was enriched in myofibrils.

Actomyosin Adenosine Triphosphatase Activity

The Ca^{2+}-stimulated actomyosin adenosine triphosphatase (ATPase) activity of the myofibrillar fraction was measured from the rate of decrease of nicotinamide adenine dinucleotide absorbance (340 nm excitation wavelength) in a reaction coupled to the pyruvate kinase and lactate dehydrogenase reactions, as previously described by our laboratory.[7] Triton X-100–extracted myofibrils were solubilized in a buffer containing the following: 25 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 7.4), 85 mM potassium methanol sulfonic acid, 3 mM $MgCl_2$, 2 mM EGTA, 10 mM sodium fluorescein, 0.5 mM dithiothreitol, 0.5 mM leupeptin. The protein concentration of the extracted myofibrils was determined using the Lowry protein assay.[12] The reaction mixture consisted of the following: 25 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 7.0), 2.7 mM $MgCl_2$, 2 mM EGTA, 10 mM sodium fluorescein, 126 mM potassium methanol sulfonic acid, and varying free calcium chloride concentrations giving free Ca^{2+} concentrations from pCa 9 to 4. The reaction mixtures were prepared using an iterative computer program to determine the amount of calcium chloride required to achieve the appropriate concentrations. The reaction mixture also contained 200 mM phosphoenolpyruvate, 10 mM nicotinamide adenine dinucleotide, 0.5 mg/ml lactate dehydrogenase, 12.5 mg/ml pyruvate kinase, and 1 ml of one of the calcium buffers (pCa 4–9) containing myofibrillar fractions. The reaction was initiated by addition of 2 mM ATP and was allowed to continue for up to 10 min (37°C), although the reaction was usually complete within 5 min. Ca^{2+}-stimulated actomyosin ATPase activity was monitored by the formation of ADP, coupled to the oxidation of nicotinamide adenine dinucleotide, and recorded by the change in absorption at 340 nm. The enzyme activity was determined from the rate of ATP hydrolysis and expressed as the percent of maximal actomyosin ATPase activity per milligram of protein.

Labeling of Myofibrils in Intact Ventricular Myocytes with [^{32}P]Orthophosphate

Phosphorylation of myofibrils in rat ventricular myocytes by [^{32}P]orthophosphate ($^{32}P_i$) was performed as previously described by our laboratory.[13] Freshly isolated ventricular myocytes were suspended in 12 ml phosphate-free HBS (5×10^5 cells/ml) and incubated with 250 μCi $^{32}P_i$ for 2 h at room temperature. The cell suspension was gently agitated and maintained under an oxygen hood throughout the duration of the $^{32}P_i$-labeling period. There was no significant loss in myocyte viability (rod-shaped cells) following the 2-h labeling period with $^{32}P_i$. After labeling, the cells were centrifuged (400 rpm) and resuspended in phosphate-free Henseleit buffer, divided into 2-ml aliquots and placed in test tubes. To ensure that the cells remained viable and maximally oxygenated throughout the incubation, all tubes were purged with 100% O_2, then placed on their sides and gently agitated for 10 min at 37°C in a shaking water bath. Myocyte viability remained greater than 75% following the 10-min incubation at 37°C in the presence of the agonists and inhibitors. Following the incubation period, the reactions were terminated, and interventions

were removed by rapidly washing the myocytes in saline (4°C, 5 ml) containing protease inhibitors (5 μg/ml pepstatin A, 10 μg/ml leupeptin, 43 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml antipain, and 5 mM EGTA) and a protein phosphatase inhibitor (0.1 μM sodium orthovanadate) immediately before suspending the pellet in 2 ml ice-cold "inhibiting buffer" (50 mM KH$_2$PO$_4$, 70 mM sodium fluorescein, and 5 mM EDTA), as described by Holroyde *et al.*[14] Myofibrils were extracted on ice for 1 h by adding 1% Triton X-100 plus the protease and phosphatase inhibitors listed above to the inhibiting buffer. Detergent-extracted myofibrils were centrifuged at 5,000g (5 min) using a microcentrifuge. The supernatant was discarded. Examination of the pellet under the light microscope indicated that it was enriched in myofibrils.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of ^{32}P$_i$-labeled cardiac myofibrils was performed on 12% slab gels, as described by Laemmli.[15] The myofibrillar pellet was solubilized and denatured in a sample preparation buffer containing 4% sodium dodecyl sulfate (SDS), 0.23 M 2-mercaptoethanol, and 0.2 M Tris(hydroxymethyl) -aminomethane hydrochloric acid (pH 6.5) at 100°C for 5 min. To standardize loading of the gels, protein determinations of the myofibrillar extracts were performed on each of the samples prior to loading the gel. Seventy-five micrograms protein was applied to each lane, and polyacrylamide gel electrophoresis was performed in the presence of 0.1% SDS at constant current (10 mAmp) overnight (16 h). Gels were dried the following day and subjected to phosphor screen autoradiography for quantitation of labeled proteins. A PhosphorImager (Molecular Dynamics, Piscataway, NJ) was used to quantify the amount of radioactivity in each band. The extent of ^{32}P$_i$ incorporation into protein bands was quantified using a commercial software package (Molecular Dynamics).

Experimental Controls and Data Analysis

Untreated controls were normalized to 100%. Increases in the phosphorylation of TnI and MLC2 in response to propofol, phorbol myristic acetate (PMA), or isoproterenol were calculated as percent increases in ^{32}P$_i$ incorporation compared with untreated, time-matched controls. Following pretreatment with the selective PKC inhibitor bisindolylmaleimide I (Bis),[16,17] inhibition of TnI and MLC2 phosphorylation by propofol, the PKC activator PMA,[18,19] or isoproterenol was calculated as percent inhibition of ^{32}P$_i$ incorporation compared with myocytes treated with these compounds. Therefore, percent inhibition refers to percent inhibition of the increase above basal phosphorylation. Results of all trials from 1 day were averaged to give a single value, so results from different experiments were

weighted equally. None of the interventions altered the morphology of the myocyte.

Subcellular Fractionation of Cardiomyocytes and Western Blot Analysis

Fractionation of cardiac myocytes was performed as previously reported.[20] Following treatment with PMA or propofol, myocytes were quickly pelleted by centrifugation (45 s at 800g) and washed with ice-cold HBS. Myocytes were resuspended in relaxing solution (4 mM MgATP, 100 mM potassium chloride, 10 mM imidazole, 2 mM EGTA, 1 mM MgCl$_2$, 1 mM paramethylsulfonic acid, 1 mM sodium orthovanadate, 10 mM benzamidine, and 0.01 mM leupeptin) containing 20% glycerol and 0.05% Triton X-100. Cell permeabilization and membrane solubilization were facilitated by sonication for 5 min on ice. A nuclear fraction was separated by centrifugation at 800g for 2 min. The supernatant was centrifuged at 100,000g for 30 min at 4°C, producing a pellet (designated as the membrane fraction) and the supernatant (designated as the cytosolic fraction).

Immunoblot analysis was performed on the cytosolic and membrane fractions. Samples were prepared for Western blots by measuring protein concentration using the Bradford method (Bio-Rad, Hercules, CA). All samples were adjusted to a protein concentration of 1–2 mg/ml in sample buffer, boiled for 5 min, and then kept at −20°C until use. Equal amounts of protein (50 μg) from each fraction were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes, as previously described by our laboratory.[13] Blots were stained with 1% Ponceau S to visualize protein bands and confirm complete transfer of proteins to blots. Nonspecific binding was blocked with Tris buffered saline with Tween 20 (TBS-T) (0.1%, v/v) in 20 mM Tris(hydroxymethyl) -aminomethane base, 137 mM sodium chloride adjusted to pH 7.6 with hydrochloric acid, containing 3% (w/v) bovine serum albumin for 1 h at room temperature. A monoclonal antibody against PKC-α was diluted 1:1,000 in TBS-T containing 1% bovine serum albumin for immunoblotting (2 h). After washing in TBS-T solution 3 times (10 min each), filters were incubated for 1 h at room temperature with horseradish-peroxidase–linked secondary antibody (ovine antimouse; 1:5,000 dilution in TBS-T containing 1% bovine serum albumin). Filters were again washed and bound antibody detected by the enhanced chemiluminescence method.

Measurement of [Ca^{2+}]$_i$ and Shortening

Simultaneous measurement of intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) and cell shortening was performed as previously described.[8] Ventricular myocytes (0.5 × 10^6 cells/ml) were incubated in HBS containing 2 μM fura-2–acetoxy methylester at room temperature for 15 min. Fura-2–loaded ventricular myocytes were placed in a temperature-regulated (30°C) chamber (Bioptechs,

Inc., Butler, PA) mounted on the stage of an Olympus IX-70 inverted fluorescence microscope (Olympus America, Lake Success, NY). The cells were superfused continuously with HBS at a flow rate of 2 ml/min and field-stimulated *via* bipolar platinum electrodes at a frequency of 0.5 Hz with a 5-ms pulse using a Grass SD9 stimulator (Grass Instruments, West Warwick, RI). Myocytes exhibiting a rod-shaped appearance with clear striations were chosen for study.

Fluorescence measurements were performed on individual myocytes using a dual-wavelength spectrofluorometer (Deltascan RFK6002; Photon Technology International, Lawrenceville, NJ) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Because calibration procedures rely on a number of assumptions, the ratio of the light intensities at the two wavelengths was used to measure qualitative changes in $[Ca^{2+}]_i$. Just before data acquisition, background fluorescence was measured and automatically subtracted from the subsequent experimental measurement. The fluorescence sampling frequency was 100 Hz, and data were collected using software from Photon Technology International.

To simultaneously monitor cell shortening, the cells were also illuminated with red light. A dichroic mirror (600-nm cutoff) in the emission path deflected the cell image through a CCD video camera (Phillips VC 62505T; Marshall Electronics, Culver City, CA) into a video-edge detector (Crescent Electronics, Sandy, UT) with 16-ms resolution. The video-edge detector was calibrated using a stage micrometer so that cell lengths during shortening and relengthening could be measured.

Analysis of $[Ca^{2+}]_i$ and Shortening Data

Fluorescence data for the $[Ca^{2+}]_i$ and shortening measurements were imported into LabVIEW (National Instruments, Austin, TX) for analysis of $[Ca^{2+}]_i$ and shortening. Myocyte length in response to field stimulation was measured (micrometers) and expressed as the change from resting cell length (twitch amplitude). Changes in twitch amplitude in response to the interventions are expressed as a percentage of baseline shortening. Contractile parameters from 15 contractions were averaged to obtain mean values at baseline and in response to the various interventions. Averaging the parameters over time minimizes beat-to-beat variation. Changes in $[Ca^{2+}]_i$ were measured as the change in the 340/380 ratio from baseline.

Experimental Protocols

Protocol 1: Effects of Propofol and Bis on Myofibrillar Actomyosin ATPase Activity. To determine whether propofol alters myofilament Ca^{2+} sensitivity, changes in actomyosin ATPase activity were measured in myofibrils isolated from control and propofol-treated (10 min) cardiomyocytes. The myocyte suspension was

divided into separate aliquots, and each aliquot was treated with Bis (1 μM) alone, propofol (30 μM) alone, or pretreatment with Bis for 10 min before addition of propofol at 37°C with gentle agitation. Neither propofol nor Bis had any effect on extracellular pH at the concentrations used in this study and did not appear to alter the assay conditions. Activity is expressed as a percentage of the maximum rate per milligram of protein.

Protocol 2: Effects of Propofol on $^{32}P_i$ Incorporation into Cardiomyocyte Contractile Proteins. To determine whether propofol alters myofibrillar protein phosphorylation, we examined the extent to which $^{32}P_i$-orthophosphate was incorporated into myofibrillar proteins isolated from control and propofol-treated cardiomyocytes. The $^{32}P_i$-labeled myocyte suspension was divided into separate aliquots, and each aliquot was pretreated with one concentration of propofol (1, 3, 10, 30, 60, and 100 μM) for 10 min with gentle agitation. Myofibrillar proteins were isolated, subjected to polyacrylamide gel electrophoresis and phosphor screen autoradiography for quantitation of the incorporated $^{32}P_i$ into contractile proteins. Data are expressed as percent increase in $^{32}P_i$ incorporation compared with control.

Protocol 3: Effects of Bis on Propofol-, PMA-, and Isoproterenol-simulated Incorporation of $^{32}P_i$ Incorporation into Cardiomyocyte Contractile Proteins. To determine whether the propofol-, PMA-, and isoproterenol-induced phosphorylation of contractile proteins in cardiomyocytes was mediated *via* PKC activation, we examined the extent to which PKC inhibition with Bis altered phosphorylation of TnI and MLC2. Cardiomyocytes were pretreated with Bis (1 μM; 10 min) prior to a 10 min exposure to propofol (30 μM), PMA (1 μM), or isoproterenol (1 μM). Myofibrillar proteins were isolated and processed as described in protocol 2.

Protocol 4: Effects of Propofol and Bis on PKC-α Translocation. To determine whether propofol causes translocation of PKC isoforms, we performed Western blot analysis on subcellular fractions of cardiomyocytes treated with PMA (1 μM), propofol alone (30 μM), or Bis (1 μM; 10 min pretreatment) plus propofol. Cytosolic and membrane fractions were prepared; proteins were separated and transferred to nitrocellulose for antibody labeling. Because our previous findings suggest a role for PKC-α in the propofol-induced changes in β-adrenoreceptor signaling in cardiomyocytes,[9] translocation of PKC-α was examined in this study. Rat brain lysate was used as a positive control for PKC-α.

Protocol 5: Effects of Propofol and Bis on the Extracellular Ca^{2+}–Shortening Relationship. To determine whether propofol alters myofilament Ca^{2+} sensitivity at the functional level (myocyte contractility) *via* a PKC-dependent pathway, we examined the dose–response curve to extracellular Ca^{2+} ($[Ca^{2+}]_o$) in the presence or absence of propofol and Bis. Baseline parameters were collected from individual myocytes for

1.5 min. Dose–response curves to $[Ca^{2+}]_o$ were performed by exchanging the buffer in the dish with a new buffer containing the desired $[Ca^{2+}]_o$. Data were acquired for 1.5 min following establishment of a new steady state. Dose–response curves to $[Ca^{2+}]_o$ were then performed in the presence of propofol (30 μM). Cells were allowed to stabilize for 5 min following each intervention. The relative contribution of PKC in mediating changes in the $[Ca^{2+}]_o$–shortening relationship was assessed by pretreating the cells (10 min) with Bis (1 μM).

Materials

Collagenase type II was obtained from Worthington Biochemical (Freehold, NJ). Propofol was acquired from Research Biochemicals International (Natick, MA) and solubilized in dimethylsulfoxide to appropriate stock concentrations. PMA, Bis, and isoproterenol were purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-100, protogel (30%, w/v) acrylamide, 0.8% (w/v) bisacrylamide stock solution, and 10X electrophoresis tank buffer (0.25 M Tris(hydroxymethyl) -aminomethane–1.92 M glycine–1% SDS) were purchased from National Diagnostics (Atlanta, GA). TEMED (N,N,N,N-tetramethylethylenediamine), protein G, 2-mercaptoethanol, ammonium persulfate, and prestained high- and low-molecular-weight markers were obtained from Biorad Laboratories, Inc. (Melville, NY). A monoclonal antibody against PKC-α was obtained from Upstate Biotechnology (Lake Placid, NY). Fura-2/AM was obtained from Texas Fluorescence Labs (Austin, TX).

Statistical Analysis

Each experimental protocol was performed on multiple myocytes from the same heart and repeated in at least four hearts. Results obtained from myocytes in each heart were averaged so that all hearts were weighted equally. Results of all trials for each experimental condition were averaged, and comparisons between groups were made by two-way analysis of variance and Bonferroni–Dunn *post hoc* test. Differences were considered statistically significant at $P < 0.05$. Results from each experimental condition were normalized to unstimulated controls, which were taken as 100%. Results are expressed as mean $\pm$ SEM.

Results

Effects of Propofol and Bis on Actomyosin ATPase Activity

To directly test the hypothesis that propofol increases myofilament Ca^{2+} sensitivity *via* a PKC-dependent pathway, we assessed the effects of propofol (30 μM) alone and following pretreatment with Bis (1 μM), on myofibrillar actomyosin ATPase activity. As previously demonstrated,[7] propofol alone caused a leftward shift in the
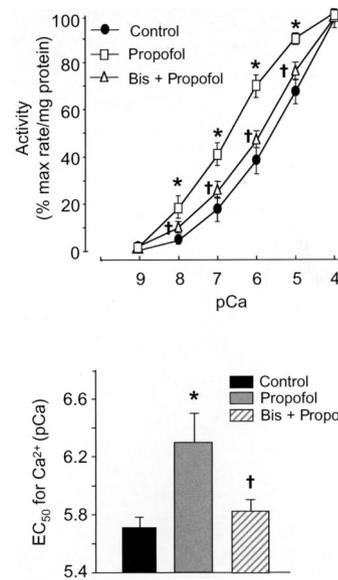

Fig. 1. (*Top*) Summarized data depicting the effects of Bis (1 μM) on the propofol-induced (30 μM) leftward shift in actomyosin ATPase activity in isolated myofibrils. (*Bottom*) Summarized data depicting the increase (*$P < 0.05$ vs. control) in the EC_{50} value for Ca^{2+} induced by propofol and inhibition of this effect (†$P < 0.05$ vs. propofol) following pretreatment with Bis.

actomyosin ATPase activation curve (fig. 1). Propofol increased the EC_{50} (pCa) value (*i.e.*, decreased the Ca^{2+} requirement) from 5.7 ± 0.1 to 6.3 ± 0.2 ($P < 0.05$). Propofol (30 μM) reduced ($P < 0.05$) the maximal activation of actomyosin ATPase (V_{max}) by $15 \pm 7\%$, from a control value of 186 ± 11 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. Pretreatment with Bis (1 μM) attenuated the propofol-induced leftward shift (EC_{50} value $= 5.8 \pm 0.1$) and reduction in V_{max} (181 ± 16 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}). Bis alone had no effect on actomyosin ATPase activity.

Effect of Propofol on $^{32}P_i$ Incorporation into Cardiomyocyte Contractile Proteins

Following a 2-h prelabeling period of endogenous pools of ATP with $^{32}P_i$, myocytes were washed and subsequently incubated with propofol for 10 min at 37°C. Autoradiography of SDS gels of myofibrils from untreated (CON) myocytes demonstrated the presence of $^{32}P_i$ incorporation in several myofibrillar proteins, including troponin T (TnT), tropomyosin, TnI, and MLC2 (fig. 2, *lane 1*). Propofol caused a dose-dependent increase in the labeling of TnI and MLC2, with peak phosphorylation levels occurring at about 60 μM for both myofibrillar proteins. Propofol (10 μM) caused a $74 \pm 13\%$ increase in the phosphorylation of TnI above baseline, whereas MLC2 phosphorylation was increased by $167 \pm 15\%$.

Effect of PKC Inhibition on Propofol-, PMA-, and Isoproterenol-induced Incorporation of $^{32}P_i$ into Cardiomyocyte Contractile Proteins

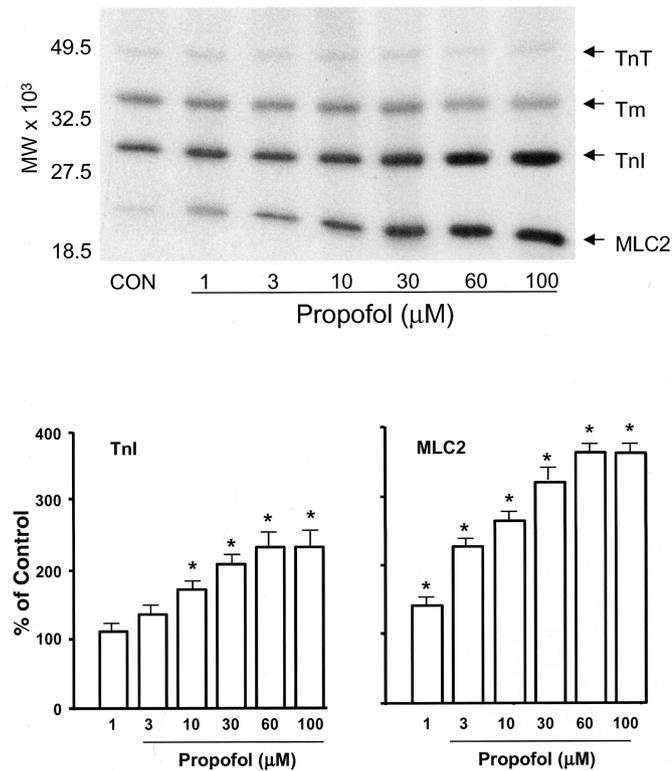To test the hypothesis that PKC was responsible for the propofol-induced phosphorylation of TnI and MLC2,

Fig. 5. Summarized data for the effects of Bis on the propofol-induced (30 μM) shift in the $[Ca^{2+}]_o$–shortening relationship. *Left*, cell shortening; *right*, 340/380 ratio. *Significant change from control ($P < 0.05$). †Significant change from propofol ($P < 0.05$). n = 15 cells/5 hearts.

Effect of Bis on the Propofol-induced Shift in the Dose–Response Curve to $[Ca^{2+}]_o$

Confirming our previous results,[8] the propofol-induced increase in myofilament Ca^{2+} sensitivity is manifested as an upward shift in the $[Ca^{2+}]_o$–shortening relationship (fig. 5). To test the hypothesis that this effect is due to PKC activation, we assessed the effect of Bis (1 μM) on the propofol-induced change in the $[Ca^{2+}]_o$–shortening relationship. Bis abolished the propofol-induced shift in the $[Ca^{2+}]_o$–shortening relationship (fig. 5).

Discussion

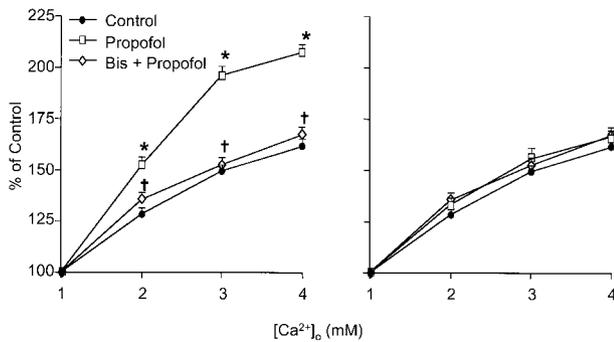This is the first study to both directly assess the role of PKC in mediating propofol-induced alterations in myofilament Ca^{2+} sensitivity and identify the extent to which propofol alters the phosphorylation state of the myofibrillar contractile proteins, TnI and MLC2. We previously demonstrated that propofol increases myofilament Ca^{2+} sensitivity and intracellular pH (pH_i) *via* activation of Na^+-H^+ exchange in rat ventricular myocytes.[7] The increase in pH_i was attributed to a propofol-induced activation of PKC. We also demonstrated that the increase in myofilament Ca^{2+} sensitivity observed in field-stimulated cardiomyocytes could be partially inhibited by ethylisopropyl amiloride, an inhibitor of Na^+-H^+ exchange. The major findings of the current study are that PKC activation mediates the propofol-induced leftward shift in the actomyosin ATPase activity curve (*i.e.*, a decrease in the amount of Ca^{2+} required for activation). In addition, propofol stimulates phosphorylation of the contractile proteins, TnI and MLC2, *via* a PKC-dependent pathway. Propofol also stimulates translocation of PKC-α from the cytosolic to membrane fraction. The phosphorylation of MLC2 by the PKC signaling pathway, in addition to a previously observed PKC-dependent increase in pH_i, likely explains the propofol-induced increase in myofilament Ca^{2+} sensitivity in rat ventricular myocytes.

Effects of Propofol and PKC Inhibition on Actomyosin ATPase Activity

The molecular basis for myocardial contractility involves an increase in $[Ca^{2+}]_i$, which in turn triggers activation of the intrinsic actomyosin ATPase associated with the cardiac myofibrils. The sensitivity of the contractile machinery to Ca^{2+} is regulated primarily by the troponin complex associated with the thin filament. It is well established that alterations in pH_i can modulate myofilament Ca^{2+} sensitivity. In addition, phosphorylation of the contractile proteins TnI, TnT, and MLC2 play a role in modulating myofilament Ca^{2+} sensitivity.[2,4,5] In our previous study, we demonstrated that myofibrils isolated from propofol-treated cardiomyocytes exhibited a leftward shift in the actomyosin ATPase activity curve compared with myofibrils obtained from untreated cells.[7] We also observed that propofol did not directly alter actomyosin ATPase activity in isolated myofibrils, indicating a requirement for a cytosolic mediator for the actions of propofol on actomyosin ATPase activity. In the current study, we observed that pretreatment of the cardiomyocytes with the PKC inhibitor, Bis, abolished the propofol-induced leftward shift in the actomyosin ATPase activity curve. These data suggest that a PKC isoform(s) likely represents the cytosolic mediator involved in the propofol-induced increase in myofilament Ca^{2+} sensitivity. These current data are consistent with other studies demonstrating that propofol and halothane stimulate purified brain PKC activity[21,22] and that PKC activation increases myofilament Ca^{2+} sensitivity in cardiac myocytes.[2-4,23]

Effect of Propofol on Phosphorylation of Contractile Proteins

Phosphorylation of contractile proteins in response to myocardial receptor activation is known to result in changes in cardiac inotropy and lusitropy mediated by alterations in myofilament responsiveness to Ca^{2+}. This is the first study to demonstrate a propofol-induced phosphorylation of TnI and MLC2 at clinically relevant concentrations. Although no stoichiometry was performed, propofol stimulated a much greater increase in MLC2 phosphorylation than that observed for TnI phosphorylation. Phosphorylation of TnI can occur *via* activation of the PKA and/or PKC signaling pathways.[5] In contrast, phosphorylation of MLC2 can occur *via* activation of myosin light chain kinase, PKC, as well as activation of PKA.[2-5] Phosphorylation of TnI is associated with a decrease in myofilament Ca^{2+} sensitivity manifested as a rightward shift in the actomyosin ATPase activity curve.[5] In contrast, phosphorylation of MLC2 is associated with an increase in actomyosin ATPase activity manifested as a leftward shift in the actomyosin ATPase activity curve[3,4] Therefore, the overall net effect of TnI and MLC2 phosphorylation on actomyosin ATPase activity, and hence myocardial performance, will be the sum

of the individual components. Because the propofol-induced phosphorylation of MLC2 was much greater than that observed for TnI, we propose that phosphorylation of MLC2, together with the increase in pH_i observed in our earlier study,[7] plays a primary role in contributing to the propofol-induced increase in myofilament Ca^{2+} sensitivity. Our next goal was to determine whether propofol-induced activation of PKC mediates the phosphorylation of contractile proteins in cardiomyocytes.

Effect of PKC Inhibition with Bis on Propofol-Induced Phosphorylation of Contractile Proteins

Previous studies have demonstrated that TnI and MLC2 are substrates for PKC-, PKA-, and MLCK-dependent phosphorylation in myocardial cells.[2,4,5] We hypothesized that PKC activation by propofol would result in the phosphorylation of myofibrillar contractile proteins. PKC activation with PMA stimulates the phosphorylation of TnI and MLC2, as previously demonstrated by our laboratory and others.[2,4,13] In addition, PKC inhibition with Bis attenuates both the propofol-induced and PMA-induced phosphorylation of TnI and MLC2, providing further support for a propofol-induced activation of PKC as the mediator of the phosphorylation of TnI and MLC2. Finally, addition of propofol and PMA together did not result in an additive effect on the phosphorylation of TnI or MLC2, indicating that both compounds are working through a common pathway involving activation of PKC. The current study also provides data demonstrating the quantitative differences in PKA-dependent phosphorylation of TnI and MLC2 compared with PKC-dependent phosphorylation. Compared with PMA- and propofol-stimulated phosphorylation of TnI and MLC2, β-adrenoreceptor activation with isoproterenol stimulated increases in TnI phosphorylation that were greater than those observed with propofol or PMA alone. In contrast to PMA and propofol, isoproterenol stimulated little increase in MLC2 phosphorylation. It should be noted that phosphorylation of TnI by PKC or PKA occurs at different sites on TnI, which may serve to fine-tune effects on actomyosin ATPase activity.[5] Specifically, PKA-mediated phosphorylation of TnI occurs on serine 23/24, leading to desensitization of the myofibrils to Ca^{2+}, whereas PKC-dependent phosphorylation of TnI occurs on serine 43/45 and/or threonine 144, resulting in an increase in the sensitivity of the myofibrils to Ca^{2+}. Therefore, PKA- and PKC-dependent phosphorylation of TnI can result in opposing effects on myofilament Ca^{2+} sensitization. Because pretreatment with Bis had no effect on β-adrenoreceptor-dependent phosphorylation but inhibited propofol- and PMA-induced phosphorylation, our results suggest that Bis is a specific inhibitor of PKC-dependent phosphorylation events in cardiac myocytes. Therefore, we suspect that propofol- and PMA-induced phosphorylation of MLC2 plays a more important role than TnI phosphorylation in determining the overall net effect of PKC activation on actomyosin ATPase activity and myocyte contractile function.

Effects of Propofol and Bis on PKC-α Translocation

Based on recent findings from our laboratory,[9] we used a monoclonal antibody to PKC-α and probed subcellular fractions (cytosolic and membrane) of propofol- and PMA-treated cardiomyocytes. Translocation of PKC-α from the cytosol to membrane fraction in response to propofol was observed, and PKC inhibition with Bis prevented the translocation. These data are consistent with a propofol-induced activation of PKC-α in rat cardiomyocytes. However, to establish a linkage between PKC-α translocation and phosphorylation of contractile proteins, it will be necessary to identify translocation of the PKC isoform to a distinct intracellular site. Propofol may activate additional PKC isoforms in parallel with PKC-α, which could have specific and selective roles in mediating the functional effects of propofol on cardiomyocyte contractility. These effects could include activation of Na^+–H^+ exchange, phosphorylation of contractile proteins, and inhibition of L-type Ca^{2+} channel activity. Further studies are required to fully elucidate these possibilities.

Effect of Bis on the Propofol-induced Shift in the $[Ca^{2+}]_o$–Shortening Relationship

If PKC activation mediates the propofol-induced shift in the $[Ca^{2+}]_o$–shortening relationship, then this effect should be blocked by inhibiting PKC with Bis. Our results indicate that inhibition of PKC with Bis attenuates the propofol-induced increase in myofilament Ca^{2+} sensitivity by more than 90%. We previously demonstrated that inhibition of Na^+–H^+ exchange with ethylisopropyl amiloride attenuated the shift in the $[Ca^{2+}]_o$–shortening relationship by approximately 50%.[7] Our current data are consistent with the idea that PKC activation results in phosphorylation of MLC2 and plays a role in mediating the propofol-induced shift in the $[Ca^{2+}]_o$–shortening relationship.

Clinical Implications

As always, extrapolation of results obtained from *in vitro* studies at the cellular level to the clinical setting can be difficult. However, it is known that PKC activation is a key mediator of ischemic and anesthetic preconditioning and cardiac protection. In addition, increases in myofilament Ca^{2+} sensitivity can partially offset the negative inotropic effects of certain agents, including propofol. Therefore, propofol may be beneficial in patients exhibiting end-stage heart failure (or other cardiomyopathies) in which Ca^{2+} overload is observed. In this patient population, propofol may increase cardiac function without further altering Ca^{2+} homeostasis, which could be beneficial to the patient.

Limitations

It is well known that propofol partitions *in vivo* between serum proteins, lipid microsomes, and into tissue. Therefore, difficulty arises in determining the precise concentration of free and active propofol at the tissue level. In the current study, we used isolated cells rather than intact tissue. Therefore, the effects of propofol may be enhanced because of reduced solute diffusion distances between the cytosol and extracellular medium. Also, this *in vitro* study only deals with intrinsic myocardial function, whereas changes in cardiac contractility *in vivo* following propofol administration also depend on a variety of other factors including venous return, afterload, and neurohumoral compensatory mechanisms. In addition, the experimental conditions (temperature, stimulation frequency, unloaded cells) used in this study do not parallel *in vivo* conditions. However, the strength of this model is that we can directly assess the effects of propofol on cellular mechanisms that regulate contractile function.

Summary

Our results provide the first direct evidence that propofol increases the sensitivity of myofibrillar actomyosin ATPase to Ca^{2+} (*i.e.*, increases myofilament Ca^{2+} sensitivity) *via* a PKC-dependent pathway. In addition, our results demonstrate that propofol stimulates phosphorylation of TnI and MLC2 and results in translocation of PKC-α from the cytosolic to the membrane fraction. It is likely that PKC-dependent myofibrillar protein phosphorylation contributes to the propofol-induced increase in myofilament Ca^{2+} sensitivity in rat ventricular myocytes.

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